

# Chapter 5

## Cytokines in Skeletal Muscle Growth and Decay

Arkadiusz Orzechowski

**Abstract** By definition, cytokines are the first messengers of intercellular communications observed among leukocytes. Numerous cytokines control immune system and biological reactions thereof, but are functionally grouped into pro- and anti-inflammatory varieties (the latter are also involved in allergic reactions). The bulk of evidence points to substantial role played by cytokines in skeletal muscle growth and wasting. Cytokines and growth factors of immune origin affect skeletal muscle growth and organ formation, regeneration, and wasting but are also produced and secreted by muscle fibers as myokines. To orchestrate skeletal muscle growth, hepatocyte growth factor/scatter factor (HGF/SF) and insulin-like growth factors (IGF-I and IGF-II) play the primary physiological role being mediated through PI3-K/Akt signaling pathway. Skeletal muscle mass is in turn controlled negatively by myostatin, a member of transforming growth factor beta (TGF- $\beta$ ) superfamily. Following muscle injury, the immune-derived cytokines are most important in activation of muscle satellite cells: tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6); with IL-8 to arrange growth/regeneration; and IL-15 to control muscle hypertrophy. Some life-threatening diseases are associated with muscle wasting featured by accelerated muscle protein breakdown. In these catabolic states, cytokines such as TNF- $\alpha$  was often reported as causal factor. Moreover, cross talk between myokines (IL-6, IL-15) and adipokines (leptin) is vital for correct metabolic interorgan relations. Thus, cytokines and growth factors exist as basic chemical signals that orchestrate skeletal muscle fate in normal and diseased states.

**Keywords** Cytokines • Growth factors • Skeletal muscle • Growth • Regeneration • Cachexia

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A. Orzechowski (✉)

Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences – SGGW, Warsaw, Poland

e-mail: [orzechowski\\_arkadiusz@wp.pl](mailto:orzechowski_arkadiusz@wp.pl)

## 5.1 Cytokines in Skeletal Muscle Myogenesis and Somatic Growth

In vertebrates skeletal muscles develop during embryonic life from paraxial mesoderm (dermomyotome, myotome) specified to the myogenic lineage by paracrine signals from surrounding tissues. Wingless-related integration sites (Wnts), sonic hedgehog (SHH), and Noggin (NOG) stimulate, whereas bone morphogenetic protein 4 (BMP4) inhibits the commitment to the myogenic lineage. *Pax-3* and *Pax-7* are paired box genes targeted and co-expressed in the myotomal cells (epithelial spheres of paraxial mesoderm) [166]. Next, sequential activation of basic helix-loop-helix transcription regulators known as myogenic regulatory factors (MRFs) determines muscle progenitors termed myoblasts. Initially, *MyoD* and/or *Myf5* gene expressions are upregulated followed by *Myogenin* and/or *MRF4* gene activation. The latter are essential for induction of muscle-specific genes and terminal differentiation of mononuclear myoblasts [16]. Some of muscle-specific gene expressions such as myosin heavy chain (MyHC) or muscle creatine kinase (MCK) are valuable skeletal muscle cell markers. Dividing myogenic cells express *MyoD/Myf5* (myoblasts); subsequently they withdraw from the cell cycle to fuse upon *Myogenin* and/or *MRF4* control. Replication of activated satellite cells is possible by elevated levels of cyclin D, whereas terminal differentiation is upregulated by *Myogenin* gene expression [144]. Finally multinucleated cell syncytium (myotubes) is formed before establishing the fully mature contracting skeletal muscle fiber. Some myoblasts do not differentiate and remain dormant (satellite cells) embedded to the plasma membrane of contracting muscle fibers. After asymmetric cell division, they form a subpopulation apt to regenerate damaged muscle [46].

The major role in postnatal skeletal muscle growth is played by the growth hormone (GH)-IGF axis. Receptors for GH are present in sarcolemma, although direct binding of GH to muscle cells has never been demonstrated. In contrary to GH, IGF-I and IGF-II are able to alter MRF expression and promote muscle growth by increasing the proliferation and the differentiation of myoblasts [4, 38]. IGFs augment muscle mass through elevated DNA and protein synthesis [121, 158]. Increased number (hyperplasia) and fiber size (hypertrophy) are attributed to activation of satellite cells that in turn supply myonuclei to existing or newly formed muscle fibers [18, 19]. The hypertrophic effect of IGF-I differs from the general meaning of hypertrophy, as it is not barely increase in cytoplasmic-to-DNA volume ratio. IGFs are upregulated in skeletal muscle undergoing regeneration [48, 102], and these cytokines ameliorate muscle progenitor cells' (MPCs) viability and myogenic potential [95, 96]. Muscle growth and regeneration controlled by IGFs have distinct time windows, IGF-I being induced before IGF-II during muscle regeneration. In addition, IGFs' activity brought about in target cells is altered by local growth factor availability modulated by IGF-binding proteins (IGFBPs) [207, 208]. Furthermore, by suppressing the expression of muscle-specific atrophy-related ubiquitin ligases, muscle RING-finger protein 1 (MuRF1) and muscle

atrophy F-Box/atrogen 1 (MAFbx/atrogen 1), IGF-I inhibits protein degradation and protects skeletal muscle mass from loss. IGF-I differs from other growth factors as it stimulates both proliferation and differentiation of muscle cells [59]. Several existing isoforms of IGF-I (IGF-IEa, IGF-IEb, MGF) suggest distinct tasks played by each isoform and give possible explanation for apparently opposite roles played by IGF-I in muscle growth. Mechano growth factor (MGF) is believed to stimulate myogenic cell proliferation, while IGF-IEa seems to be decisive for terminal differentiation and muscle cell fusion [216]. It is less clear how IGF-II affects skeletal muscle growth, even though it is known that it acts on target cells through IGF-IR (no functional IGF-IIR was found) by autocrine and/or paracrine route. IGF-II was reported to accelerate terminal differentiation of C2C12 murine myoblasts in vitro and has some importance in prenatal skeletal muscle development [161]. Thus, IGF-I and IGF-II activate distinct signaling cascades, with IGF-II eliciting a stronger differentiation effect correlated with downregulation of  $G_{\alpha 12}$  protein [160]. Insulin, the main anabolic hormone in postnatal life, has numerous actions on target cells with one that mimics IGF-I-dependent skeletal muscle growth-promoting effect. Similar to IGF-I, insulin encourages skeletal myogenesis through activation of membrane receptor-associated phosphatidylinositol 3-kinase PI3-K/Akt signaling cascade that in turn inhibits glycogen synthase kinase 3-beta GSK-3 $\beta$  and forkhead box protein O1 (FoxO1) [104].

## 5.2 Skeletal Muscle Repair and Regeneration

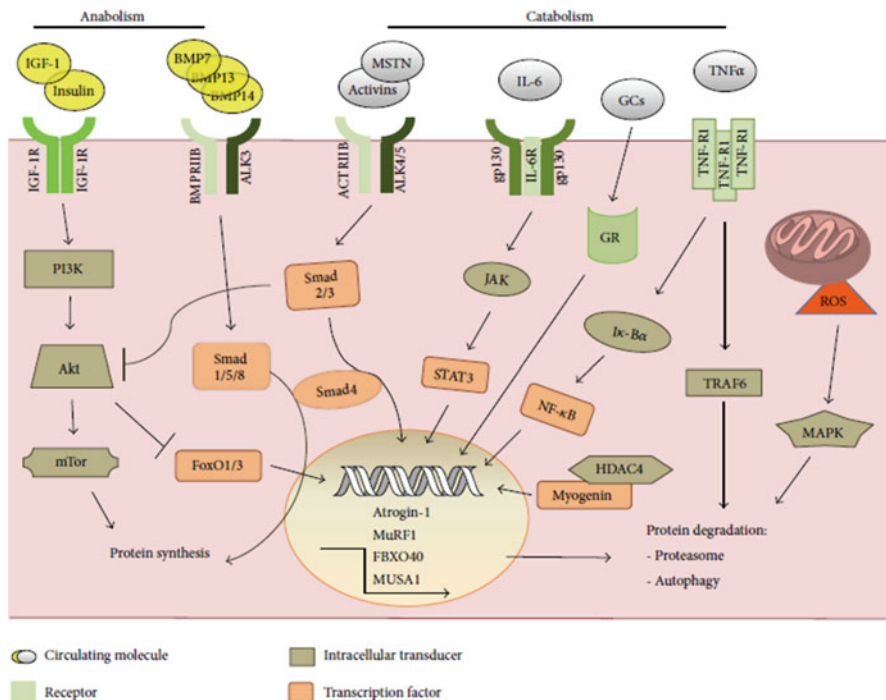
Regardless of injurious agent, any severe skeletal muscle damage starts the series of events that recapitulate myogenesis. Skeletal muscle regeneration is a highly coordinated process that absorbs adult muscle satellite cells to proliferate and differentiate. Following muscle injury, several biologically active substances are released, such as molecules from the injured fibers, soluble factors from connective tissue, and, finally, cytokines from infiltrating leukocytes. Some of them have been identified as trophic factors: fibroblast growth factor (FGF) and transforming growth factor beta (TGF- $\beta$ ) families, IGFs, hepatocyte growth factor/scatter factor (HGF/SF), tumor necrosis factor alpha (TNF- $\alpha$ ), ciliary neurotrophic factor IL (interleukin)-6 (IL-6), and leukemia inhibitory factor (LIF). There are also neural-derived low molecular weight components including nitric oxide (NO) and ATP. Not only extracellularly released factors, but cell-cell and cell-extracellular matrix (ECM) communications are decisive to instigate muscle repair [49, 101, 182]. Disruption of muscle fiber integrity (necrosis) is critically important in the inflammatory response and recruitment of satellite cells to the site of injury (degeneration phase). Disturbed calcium homeostasis activates phospholipase A<sub>2</sub> with arachidonic acid being converted to prostaglandins and thromboxanes. Sarcoplasmic proteins released through discontinued sarcolemma, as well as inflammatory mediators, are believed to be chemoattractants for resident-activated mononucleated inflammatory and muscle satellite cells (regeneration phase).

Next, chemotactic signals attract circulating inflammatory cells. Reconstruction of injured muscle is a highly orchestrated process driven by chemical signals that grant the balance between pro- and anti-inflammatory factors. To fully resolve damage with newly formed or reconstituted muscle fibers, the kinetics of order of each phase (degeneration vs. regeneration) must be precisely synchronized. The magnitude of inflammatory reaction is highest within 1–4 days after muscle injury and lasts for another week or more [34]. Finally, after successive 3 weeks, skeletal muscle is fully restored morphologically and functionally. The exact timetable of regeneration is based on the observations obtained from laboratory animal experiments carried out after intramuscular myotoxin injections [42, 75, 76], crushing and/or freezing the muscle [98, 99], single muscle autologous transplantation, or after repeated bouts of eccentric exercise [15]. Alternatively, animal models of skeletal muscle dystrophies were in extensive use as they give opportunity to monitor the degeneration/regeneration process continuously. The *mdx* mouse line, i.e., the animal model of human Duchenne muscular dystrophy (DMD), is caused by deficiency of functional dystrophin protein, a fundamental component of dystrophin-glycoprotein complex (DGC). Truncated form of dystrophin renders skeletal muscle highly susceptible to contraction-induced injury as links between cytoskeleton and ECM are weak and during exercise sarcolemma is ruptured leading to myofiber necrosis [31]. From this and other mouse models established to study consequences of skeletal muscle damage, it is clear that, e.g., fibroblast growth factor (FGF) limits the efficiency of muscle regeneration [12]. Several FGFs have been shown as potent MPC mitogens and inhibitors of differentiation [4, 47, 70, 83, 98, 99, 112, 214]. Apparently, the main task of FGFs is to expand the MPC compartment. Among FGFs, the FGF-6 isoform is of particular interest, as this growth factor is specific for skeletal muscle and it is elevated during regeneration phase [43, 60, 83]. Similarly to FGF-6, the mitogenic effect was observed for FGF-2 and FGF-4 indicating possible redundancy among FGFs. Although FGFs stimulate satellite cell proliferation and muscle regeneration, the exact nature of FGF-induced skeletal muscle regeneration is still debated and some authors even point to improved revascularization as a cause, as FGFs are also highly angiogenic [100]. Physiological effects of FGFs are mediated through cognate transmembrane FGF receptors (FGFR 1–4) with the FGF-1R known to be the most prominent in skeletal muscle cells [162]. FGFRs possess intrinsic tyrosine kinase domain typical to mediate trophic actions of cytokines and growth factors. One has to admit that picture is more complex as ECM limits the access of several molecules to the plasma membrane receptors. Thus, it is obvious that availability of FGFs and other cytokines to regulate skeletal muscle growth and regeneration is altered by the composition of ECM. Heparan sulfate proteoglycans (HSPGs) are the most important for local modulation of signals from muscle and non-muscle cells. The synergy between FGFs and HGF/SF in MPC activation is believed to be dependent on HSPGs, as heparan sulfate assists in c-Met (tyrosine kinase domain) transmembrane receptor activation and amplifies downstream cellular signaling pathway [154]. For FGFRs

the most potent HSPGs are syndecans because syndecan-3 and syndecan-4 were identified on membrane surfaces of dormant and stimulated satellite cells [39]. Thus, MPCs are prepared by ECM for early activation by FGFs and/or HGF/SF, and changes in ECM composition (especially protein sulfation) may considerably impair MPC activation. With regard to HGF/SF, this growth factor is known to play substantial role in primary and secondary organogenesis in prenatal as well as organ regeneration in postnatal life [122, 123, 219]. Injured or crushed muscle is a rich source of HGF/SF, as both HGF transcript and the peptide are elevated at the onset of muscle regeneration [185]. It is not only a potent mitogen related to serine protease plasminogen (precursor peptide has a catalytic inactive serine protease homology domain which is cleaved through proteases like plasminogen activator), but it also stimulates morphogenesis and cell migration (scattering) [163]. HGF/SF is the ligand for proto-oncogene c-Met, plasma transmembrane receptor that contains the tyrosine kinase domain liable for autophosphorylation on tyrosine residues [118]. It plays dual role in myogenesis, as it encourages muscle satellite cells to divide while it mitigates their subsequent differentiation and fusion [64]. MPCs sense HGF/SF merely at the initial phase of muscle regeneration as exogenous injection of this peptide do not improve muscle repair at later stages [118]. Similarly, HGF/SF immunostaining is reduced at specimens obtained from damaged muscle with time after injury [185]. c-Met exposed to HGF/SF is able to activate MPCs through several signaling pathways (Gab1/Shp2/Ras/Raf/Erk, Grb2/Sos/Ras/Raf/Erk, Gab1/PI3K/Akt, Gab1/Crk/C3G/Rap1) which regulate cell proliferation/morphogenesis, survival, and actin reorganization, respectively. Pleiotropic effects of HGF/SF lead to increased MPC population and backing up satellite cell migration that in overall provide optimal myoblast density to the site of muscle injury. Besides HGF/SF secreted by MPCs in an autocrine route [165], certain pool is available from the ECM, especially when basal lamina is disrupted [186] or NO is released by nNOS at the motor end plates [13]. Other organs can also contribute to skeletal muscle regeneration, e.g., muscle damage stimulates spleen to secrete HGF/SF in rats [183]. Altogether, HGF/SF comes out to be a primary mitogen and the most important growth factor involved in skeletal muscle regeneration (Fig. 5.1).

### 5.3 Muscle Injury and Immune Cells

Even though molecular regulation of myogenesis and skeletal muscle regeneration are almost indistinguishable, the immune response is observed solely in harmed muscle. Immune cells are of myeloid origin and amount to high numbers ( $10^5/\text{mm}^3$ ) in damaged muscle site. Immune cells actively secrete numerous cytokines and growth factors in order to modulate own inflammatory activity, and they affect the viability and transcriptional activities of regenerating muscle cells [190]. Most of the biological effects observed in the immune system are mediated by cytokines divided



**Fig. 5.1** Humoral mediators and associated pathways drive anabolic and catabolic responses in the skeletal muscle (Costamagna et al. [40])

into two groups: one represented by pro-inflammatory activity and second suited by anti-inflammatory action. Nowadays, there is a strong belief that the balance between pro- and anti-inflammatory cytokines is essential for accurate immune response to injury. Although expression of myogenic genes occurs in the absence of myeloid cells, the latter can control the extent and timing of expression. It is obvious that some cytokines endorse muscle satellite cell proliferation in vitro [29, 109].

T lymphocytes embody major source of cytokines with CD4-expressing T lymphocytes (CD4<sup>+</sup>) also known as helper cells, the most useful cytokine producers. CD4<sup>+</sup> T lymphocytes could be further subdivided into Th1 and Th2, while the cytokines they secrete are termed Th1-type (pro-inflammatory such as interferon-γ, TNF-α, interleukin 1β) and Th2-type (anti-inflammatory such as interleukins 3, 5, 10, and 13), respectively. Inflammatory lesion in skeletal muscle begins the fast infiltration with Ly6C<sup>+</sup>/F4/80<sup>-</sup> neutrophils during acute phase of inflammation (the second hour) peaking between 6 and 24 h postinjury. This phase ends up with phagocytic CD68<sup>+</sup> macrophages of M1 phenotype that rise in number between 24 and 48 h postinjury. M1 macrophages decline sharply 2 days postinjury, and this decline heralds the infiltration of non-phagocytic CD206-expressing macrophages of M2 phenotype (M2a, M2b, M2c) [170, 191]. The induction of MRF in skeletal

muscle progenitors is in striking coincidence with the release of inflammatory and vascular mediators by immune cells. First, the degree of inflammatory reaction affects regenerating muscle, with greater injury observed in wide-ranging infiltration by neutrophils and M1 macrophages. Free radicals (oxygen, nitrogen, and chlorate species) released by neutrophils and M1 macrophages lead to plasma membrane degradation (lipid peroxidation) and an extracellular debris for phagocytosis [27]. Thus, reductions in phagocyte-mediated damage facilitate and accelerate muscle regeneration [220].

One must bear in mind that innate immune response begins with TNF- $\alpha$ - and interferon- $\gamma$ -driven activation of macrophages. These cells acquire M1 pro-inflammatory phenotype with vast generation of nitric oxide (NO) by inducible form of nitric oxide synthase (iNOS). By the way, other destructive nitrogen species such as peroxynitrite could come out [201]. Importantly, M1 macrophages express CD68, the receptor for oxidized LDLs which in turn stimulates phagocytosis and secretion of pro-inflammatory cytokines [153].

TNF- $\alpha$  plays a critical role in inflammation as this is the first cytokine released by neutrophils and M1 macrophages to trigger production of other Th1 cytokines. Besides, TNF- $\alpha$  was frequently reported to encourage skeletal muscle regeneration at early stage following acute injury as TNF- $\alpha$  null mutants and TNF- $\alpha$  receptor mutants had lower expression levels of MyoD and MEF-2 than wild-type controls [35, 204]. In differentiating C2C12 myotubes, TNF- $\alpha$  administration augmented cell growth [103], whereas it inhibited MyHC IIA protein expression in differentiated myotubes [145, 146]. Apparently, muscle cell fusion is inhibited, whereas myoblasts proliferation is stimulated by TNF- $\alpha$  that might trigger network of complex intracellular signaling pathways engaging NF- $\kappa$ B, signal transducer and activator of transcription (STAT-1 $\alpha$ ) and other transcription factors [120, 145, 146]. NF- $\kappa$ B activation by alternative pathway plays important function in skeletal muscle, as it promotes the expression and stability of cyclin D1 leading to increased cell proliferation and inhibition of differentiation [73]. Another explanation of NF- $\kappa$ B-dependent skeletal muscle growth retardation comes from the observation that MyoD and MyoD mRNA template are NF- $\kappa$ B targets for degradation [91]. Finally, NF- $\kappa$ B was shown to bind skeletal muscle transcriptional repressor, namely, YY1 which depresses activation of muscle-specific genes [203]. Thus, the exact role of TNF- $\alpha$  in muscle growth and regeneration varies on the degree of muscle damage, particular phase (transition from early to terminal differentiation), and cellular target such as NF- $\kappa$ B [218]. Although TNF- $\alpha$  levels peak at 24 h postinjury, this cytokine levels remain elevated up to 2 weeks following acute muscle injury which advocates principal role of TNF- $\alpha$  played in skeletal muscle repair [204]. It also points to TNF- $\alpha$  targets other than NF- $\kappa$ B muscle regulatory pathways, possibly p38 MAP kinase signaling [221]. Blocking the p38 kinase pathway resulted in diminished myogenesis and increased NF- $\kappa$ B activity in C2C12 myotubes [90]. TNF- $\alpha$ -dependent effects on muscle growth/regeneration are also associated with its chemoattractant attribute to stimulate directional migration of myoblasts and satellite cells [195]. Presumably, when TNF- $\alpha$  is released from polymorphonuclear

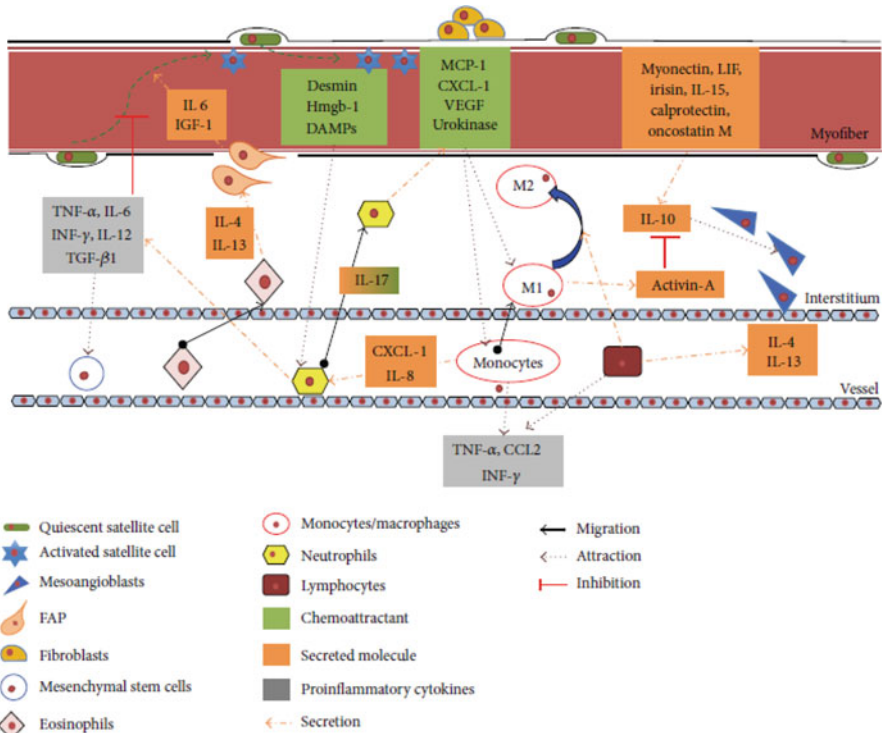
cells (PMCs) and M1 macrophages at the site of injury, it is chemical attractant for muscle stem and progenitor cells, by sensing this cytokine peel direction where the cell damage is to be compensated. Chemoattraction can be inhibited by deletion of TNF- $\alpha$  with specific antibodies or by the suppression of M1 macrophages [106, 115]. Elevated muscle protein loss is another important product of TNF- $\alpha$  activity regardless of circumstances. It is now well established that this effect of TNF- $\alpha$  is associated with induction of *FBXO32/Atrogin1* gene and its expression product atrogin-1 protein [24, 145, 146] mediated by p38 MAPK [103]. Apparently, TNF- $\alpha$  plays two opposite roles in muscle repair, one related to degenerative phase where it stimulates proliferation and migration of muscle stem and progenitor cells and the second where it holds back regenerative phase by impaired muscle cell fusion and accelerated protein decay [68, 145, 146].

Immune, type II, or  $\gamma$ -interferon (IFN- $\gamma$ ) is secreted by thymus-derived (T) cells under certain conditions of activation and by natural killer (NK) cells. IFN- $\gamma$  controls several aspects of the immune response, comprising stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through class I and class II major histocompatibility complex (MHC) molecules, or by orchestration of leukocyte-endothelium interactions linked to cell proliferation and apoptosis [25].

IFN- $\gamma$  is also another pro-inflammatory cytokine of Th1 series widely known from its pleiotropic activity taking in muscle growth and repair [36, 61]. It is worth noting, IFN- $\gamma$  cooperates with TNF- $\alpha$  in many aspects of initial adaptation to skeletal muscle injury [10, 145, 146, 194, 207, 208]. Furthermore, IFN- $\gamma$  and TNF- $\alpha$  relations are also evident during muscle cell differentiation when muscle fibers grow up and develop [145, 146, 168]. While early stages of skeletal myogenesis are apparently stimulated by both cytokines (augmented proliferation), later phases are markedly hampered, with noticed ATP-dependent proteolysis associated with E3 ubiquitin ligases atrogin-1/MAFbx and MuRF1 [145, 146]. The latter muscle-specific RING-finger protein 1 ligase and MAFbx are indicated as important IFN- $\gamma$  and TNF- $\alpha$  targets in cancer cachexia [1]. Transcriptome analysis of differentiating C2C12 muscle cells revealed that IFN- $\gamma$  affects the expression of several genes. The cytokine promoted cytokine/growth factor expression, cell proliferation, and migration but impaired muscle cell differentiation [72]. Presumably, the implementation of such a variety of effects by a single cytokine is achieved by complex patterns of skeletal muscle cell-specific gene regulation with IFN- $\gamma$  response regulated by interaction with responses to other cytokines including TNF- $\alpha$ .

IFN- $\gamma$  blocks the secretion of Th2 cytokines bulk which inhibits the inflammatory response. Thus, IFN- $\gamma$  probably upholds inflammatory response of injured muscle in order to postpone tissue repair unless the population of muscle progenitor cells is sufficient for muscle regeneration. Th2 cytokines represented by IL-4, IL-10, and IL-13 stimulate M2 macrophages to take over M1 macrophages [69]. There are several subcategories of M2 macrophages indicated by small letters (M2a, M2b, M2c) (Fig. 5.2).





**Fig. 5.2** Secreted molecules and paracrine effects from resident and circulating cells involved in skeletal muscle inflammation (Costamagna et al. [40])

### 5.4 Skeletal Muscle as an Important Source of IL-6

Myokines, a term used to describe a group of cytokines produced by skeletal muscle during and following exercise, point to a brand new role played by this organ [138]. Except MGF, remaining myokines are identical to previously described and identified leukocytes (IL-6, IL-1, interleukin 1 receptor antagonist (IL-1ra), IL-8, macrophage inflammatory protein  $\alpha$  and  $\beta$  (MIP-1 $\alpha$  and MIP-1 $\beta$ ), TNF- $\alpha$ ). Nowadays, it is widely recognized that strenuous prolonged exercise, e.g., marathon run, causes huge increase in the concentration of several pro- and anti-inflammatory cytokines in the peripheral blood [51, 52, 138]. Previously, it was well established that physical exercise stimulates the leukocytosis (increased number of leukocytes in peripheral blood). However, the origin of myokines is different from immune cells as both transcripts and proteins concentrate in skeletal muscles [66, 86, 176, 179]. The most prominent among myokines is IL-6 for the reason that following exercise this cytokine basal plasma levels may increase up to 100-fold

[58, 132, 135, 140–142]. The peak IL-6 plasma concentration is observed at the end of the exercise or soon after [56, 57, 58, 132]. For a long time, IL-6 has been considered as classical inflammatory cytokine suggesting that muscle fiber injuries promote almost exponential raise of the cytokine plasma level during a bout of heavy exercise. This assumption has no evidence as increase in IL-6 is observed in both non-damaging (concentric) [211] and damaging (eccentric) skeletal muscle contractions [107]. What is the role of IL-6 and why this myokine is that much upregulated in exercised skeletal muscle? Since IL-6 release by skeletal muscle is proportional to the muscle mass, exercise intensity, and duration (twofold increase in IL-6 level was observed after 6 min, tenfold after 2 h, and 100-fold after 6 h of running), there is presumption that this myokine is of paramount importance for homeostasis in contracting muscles, as resting muscles exposed to identical hormonal milieu do not release IL-6 [78]. Fluorescence-activated cell sorting (FACS) of peripheral monocytes isolated from blood samples of cycling or running athletes did not demonstrate any significant changes in IL-6 even during prolonged exercise [173–175]. These and other experiments show that contracting skeletal muscle is the major source of IL-6 in the peripheral blood in response to exercise and that muscle fibers but not myoblasts, endothelial cells, fibroblasts, or smooth muscle cells account for the systemic increase of plasma IL-6 [77, 86, 108, 143]. Interestingly, exercise-induced increase in IL-6 release from the exercised legs (based on arteriovenous difference) could be blocked completely by the supplementation of vitamins C and E for 4 weeks prior to the challenge [56, 57]. It appears that *IL-6* gene activation is redox mediated in skeletal muscles following exercise as antioxidants abolish this effect. Ablation of redox stimulus was also observed in differentiating myoblasts following vitamin C preincubation [131]. Given that IL-6 derived from skeletal muscle is the normal physiologic response to exercise, one may ask about its role and significance. IL-6 appears to be involved in glucose homeostasis [181, 66], and IL-6 response to exercise is dependent on pre-exercise skeletal muscle glycogen content [176]. Moreover, carbohydrate supplementation attenuates the increase of plasma IL-6 of skeletal muscle origin [174, 175]. In addition, IL-6 infusion is accompanied by extensive lipolysis and accelerated fat oxidation pointing to its metabolic rather than immunologic function [199]. Nowadays, the consistent findings indicating IL-6 secretion from exercised muscles in the absence of noticeable inflammatory markers designate this cytokine as a master switch that stimulates utilization of neutral fat when carbohydrates are in shortage. With respect to IL-6, skeletal muscle is prompted to transcriptional, translational, and secretory activities in reaction to rise in sarcoplasmic  $\text{Ca}^{2+}$  level during repetitive contractions. Importantly, the effect is calcineurin/nuclear factor of activated T-cell (NFAT) dependent (inhibited by cyclosporin A and potentiated by ionomycin). Sustained intracellular  $\text{Ca}^{2+}$  is a well-known calcineurin activator which is calcium- and calmodulin-dependent serine/threonine protein phosphatase upstream to NFAT [62]. Furthermore, *IL-6* gene expression at transcriptional and translational levels is controlled by calcineurin/NFAT in cultured muscle cells [86]. NFAT may be rephosphorylated by NFAT kinases like glycogen synthase

kinase (GSK-3 $\beta$ ) which makes NFAT inactive and withdrawn from the nucleus [54]. We observed critical role played by GSK-3 $\beta$  in myogenesis, as both insulin and metabolic inhibitors of this kinase led to ameliorated muscle fiber formation [104]. Strict pro-inflammatory TNF- $\alpha$  is repressed in the same circumstances (exercised muscle) demonstrating that IL-6 and TNF- $\alpha$  are regulated differently in skeletal muscle cells by altered Ca<sup>2+</sup> levels [84]. In addition, other possible signaling pathways explain IL-6 increase in exercised skeletal muscle. Abundantly expressed neuronal NO synthase isoform (nNOS) points toward NO production within contracting skeletal muscles with cGMP and/or nitrosative mechanisms leading to IL-6 production [93, 94, 156, 167]. Bulk of evidence collected from the experiments with NO donors showed increase in IL-6 mRNA content and IL-6 release from skeletal muscles challenged with NO, whereas opposite response was noted upon the use of nNOS inhibitors [178]. Nuclear factor kappa light chain enhancer of activated B cells (NF- $\kappa$ B) is another candidate as it is redox-sensitive transcription factor, while trained skeletal muscle is a rich source of reactive oxygen species (ROS) [167]. Antioxidants were mentioned as IL-6 repressors [189, 200]; thus it is very likely that ROS formation in skeletal muscle following exercise causes IL-6 release in a NF- $\kappa$ B-dependent manner. In order to position NF- $\kappa$ B in the redox-dependent cell signaling, nonsteroidal anti-inflammatory drugs (NSAIDs) were used as NF- $\kappa$ B inhibitors [88]. Actually, indomethacin diminishes the exercise-induced increase of IL-6 further supporting the notion of causal relationship between skeletal muscle contractions, NF- $\kappa$ B and IL-6 synthesis [155]. Moreover, basal IL-6 plasma concentrations are highly correlated with the skeletal muscle training, being lower in trained vs. non-trained individuals [32, 137]. Despite the marked rise in IL-6 mRNA during exercise, the long-term training (1 h, 5 times a week for 10 weeks) led to almost ten times lower IL-6 mRNA content in exercised skeletal muscles [56, 57]. Concomitantly IL-6R mRNA content was almost doubled in trained skeletal muscle [85]. It reveals the nature of feedback mechanism of IL-6 myokine; while plasma IL-6 is apparently downregulated by training, the muscular expression of the IL-6R is upregulated at the same time to augment IL-6 sensing by muscle fibers. The last but probably not the least, signaling pathway activated in skeletal muscle during contraction is p38 MAPK known as stress-activated protein kinase [21]. In fact, fatigued muscle with reduced glycogen content has been found to boost the p38 MAPK activity and its nuclear translocation [33]. Moreover, nuclear p38 MAPK phosphorylation through MAPK kinase (MKK-3 and MKK-6) is known to activate *IL-6* gene since inhibition of p38 MAPK results in loss of the IL-6 mRNA transcriptional control [33]. For years, p38 MAPK was shown to be essential for muscle fiber formation in myogenesis; thus its additional control of *IL-6* gene brings the role of the kinase updated [41, 90, 210]. It is very likely that the downstream targets of p38 MAPK, ATF-2, and Elk1 may participate in regulating the expression of *IL-6* gene, owing to the fact that ATF-2 is a subunit of the AP-1 heterodimer (Jun:ATF) [198], while Elk1 is a member of the Ets superfamily of transcription factors [217].

## 5.5 Cytokines Negatively Targeting Skeletal Muscle

Chronic systemic inflammation (CSI) is a cause of cardiometabolic syndrome (CMS) associated with higher risk of cardiovascular disease, type 2 diabetes, and muscle cachexia (muscle wasting) [44, 53, 141, 142, 149, 157, 192, 209]. Two- to fourfold rise in plasma concentrations of pro- and anti-inflammatory cytokines and acute-phase proteins are regarded as a common attribute of CSI [28]. Regular exercise prevents CMS almost certainly through the elevated levels of anti-inflammatory cytokines [IL-1ra, soluble TNF- $\alpha$  receptors (sTNF-R), and IL-10] which in turn override inflammatory response [134, 136, 139]. Throughout CSI several pro-inflammatory cytokines are elevated in blood plasma, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, even though TNF- $\alpha$  plays the central role in insulin desensitization of skeletal muscle [147, 148, 196]. Insulin resistance results in marked decline of its anabolic effect and accelerated loss of skeletal muscle mass typical for type 2 diabetes [147, 148]. A bulk of evidence confirms that TNF- $\alpha$ -induced insulin resistance is associated with NF- $\kappa$ B and STAT-1 $\alpha$  activation and inhibition of PI3-K/Akt signaling pathway [26, 145, 146]. Thus, TNF- $\alpha$  represents the causal factor of insulin resistance [147]. It appears that the role of IL-6 in muscle decays as pro- or anti-inflammatory cytokine is less clear as indicated by conflicting data [141, 142, 149, 172, 177]. Observations indicate that IL-6-deficient animals (knockout mice) have higher levels of circulating TNF- $\alpha$  [119], while IL-6 inhibits TNF- $\alpha$  formation [55, 110]. Additionally, IL-6 stimulates formation of anti-inflammatory cytokines IL-1ra and IL-10 [177]. IL-1ra is of particular importance as it weakens inflammatory response being the efficient blocker of IL-1 $\alpha$  and IL-1 $\beta$  binding to cognate receptors [45]. Once it is established that IL-6 has anti-inflammatory rather than pro-inflammatory characteristics, the rationale for protective role of physical activity against muscle wasting is to be revealed. Nonetheless, there is no direct relationship between exercise and reduced CSI although several studies have reported improved status of patients with CMS [67, 180]. To decipher the physiological role of IL-6, further studies are needed, as IL-6 levels are augmented both in patients with metabolic disease featured by insulin resistance and hyperlipidemia [37, 129] and after strenuous exercise which improves insulin-stimulated glucose uptake and plasma lipid profile [212]. This actually contradicting observations might be explained by simultaneous alterations in TNF- $\alpha$  concentration as increased TNF- $\alpha$  and decreased IL-6 transcription lead to higher incidence of diabetes [89]; however, the exact etiology of the CMS has at least few scenarios including elevated IL-6 as a systemic feedback mechanism to adipocyte-secreted TNF- $\alpha$  or alternative route to sensitize skeletal muscle to insulin or perhaps it results from “IL-6 resistance.” None of these hypotheses have sufficient substantiation.

For decades TGF- $\beta$  superfamily of cytokines (growth factors) has been acknowledged as inhibitors of both myoblasts proliferation and differentiation [3, 4, 23, 79]. Among several members myostatin (MSTN) also identified as growth and differentiation factor-8 (GDF-8) attracts special interest as *MSTN* knockout mice have unusually large shoulder and hip muscles [114, 184, 222]. “Loss of function” muta-

tions in *MSTN* gene were demonstrated to enlarge skeletal muscles by increased number and size of muscle fibers in other species including human beings [71, 81, 82, 113, 164]. When secreted, MSTN protein decreases myoblast proliferation by stimulating p21 protein, a well-known cyclin-dependent kinase Cdk2 inhibitor [80, 187, 188]. Cdk2 in turn inhibits G1 to S phase transition. Molecular mechanism of MSTN activity seems to hamper satellite cell proliferation through activin type II receptor (Act RIIB) as dominant negative form of Act RIIB in transgenic mouse lines exhibited dramatic increases in muscle mass comparable to those seen in *MSTN* knockout mice [97]. MRF genes are transcriptionally repressed by MSTN because signal transduction from Act RIIB directly activates Smad2/3 proteins with resultant fall in *MyoD* and *myogenin* gene expression [92, 105]. Moreover, MSTN is associated with increased Smad2/3 phosphorylation levels and decline in *MyoD* and *myogenin* gene transcription [206]. Furthermore, satellite cell proliferation is noticeably elevated in *MSTN*-null mice [111]. MSTN is produced by skeletal muscle and adipose tissue [6], even though its expression levels are subject to regulation following muscle damage [215]. Fasting and glucocorticoids were indicated as possible *MSTN* gene incentives [11, 159], and both C/EBP- $\delta$  and fewer miR-27a/b seem to mediate the respective muscle wasting effects [5, 8]. Additionally, the FoxO1 and TGF- $\beta$ /Smad3, MyoD, and JNK/p38 kinase pathways are involved in MSTN secretion [9, 74, 169]. The former, FoxO1-dependent muscle atrophy results from insulin resistance, as insulin inhibits FoxO1 in skeletal muscle cells [104]. Additionally, constitutive FoxO1 activity causes *MSTN* gene disinhibition [9] in insulin-resistant states being followed by elevated MSTN expression [7]. In contrast, *MSTN* is downregulated during muscle regeneration and its levels are returned to normal in mature fibers [116, 193, 197, 222]. To some extent, there is also contribution of MSTN to aberrant regeneration in *mdx* mice (animal model of Duchenne muscle dystrophy) as improved muscle regeneration was observed in *mdx:MSTN*<sup>-/-</sup> mice [202].

## 5.6 Myogenic Myokines

Different cytokines are produced and released by skeletal muscle, while the exact role played by each is not fully elucidated. Despite IL-6 which is fairly well described in the context of skeletal muscle as endocrine organ, IL-8 is acknowledged as potent chemoattractant for neutrophils during acute phase of inflammatory reaction [17]. IL-8 belongs to distinct family of CXC chemokines where the acronym's C stands for cysteine while X is any amino acid that separates two cysteine residues at the NH<sub>2</sub> terminus. Interestingly, IL-8 plasma concentration is altered by physical activity but barely moderate-to-exhaustive eccentric exercise rouses *IL-8* gene expression in skeletal muscle and its blood plasma levels [125, 126]. A number of reports corroborated elevated IL-8 plasma levels following training during and after marathon run [127, 128, 133]. While accurate physiological

IL-8 function in skeletal muscle is not known, some authors ponder IL-8 as locally acting and associated with mild inflammatory response associated with single muscle fiber damage. This assumption is substantiated by the miniature IL-8 observed during and following concentric muscle contractions that in general are not injurious to skeletal muscle. Some studies indicate that IL-8 is angiogenic. Summing up, IL-8 by binding to its receptor CXCR1 induces chemotactic effect, whereas it stimulates endothelial epithelium for neovascularization through CXCR2 [20, 87, 130].

Besides *IL-6* and *IL-8*, also *IL-15* gene was shown to be induced in skeletal muscle during strength training [124]. The IL-15 signaling peptide acts on target cells by attaching to heterotrimeric membrane receptors (IL-15R $\alpha\beta\gamma$ ) with Janus kinases (JAK1 and 3) and STAT-3 and STAT-5 [65]. Since IL-15 cytokine is present as intracellular and secretory form, its detection is not easy carrying inconsistent reports [124, 125, 132, 135]. Anyway, several “in vitro” studies carried out on myogenic cells revealed IL-15 as anabolic factor capable to increase MyHC in differentiating muscle cells autonomously from IGFs [63, 151]. Similarly, this cytokine was demonstrated to have anabolic effects on skeletal muscle “in vivo” [150]. Furthermore, IL-15 participates in cross talk between muscle fibers and adipocytes [14]. It reduces adipose tissue mass with concurrent skeletal muscle overgrowth [30], and this is a unique muscle-to-fat endocrine axis with high therapeutic potential to combat muscle wasting in cachexia. The distinctive function of IL-15 is manifested in fully differentiated muscle cells where this cytokine represses proteolysis in muscle cachexia [150]. Accordingly, the IL-15 mRNA was markedly upregulated in differentiated skeletal myotubes but not undifferentiated myoblasts [152]. Summing up, IL-15 could be regarded as skeletal muscle growth-promoting myokine underestimated in prevention of muscle wasting.

## 5.7 Adipokines in Skeletal Muscle Growth

There is more than 30 different adipokines produced by adipocytes, making adipose tissue a significant source of regulatory factors with some being essential for fat-to-muscle communication. Leptin peptide, a “satiety hormone” is probably the most interesting amongst adipokines. It acts on target cells similarly to IL-6 as both leptin receptor (LRb) and IL-6 receptor (gp130R $\beta$ ) share a sequence homology liable for JAK recruitment and STAT phosphorylation needed for gene regulation although LRb and gp130R $\beta$  are ligand specific without cross-reactivity [50]. Can a common signaling pathway for leptin and IL-6 evoke distinct cellular responses? The answer is yes, as IL-6 binds initially to IL-6R $\alpha$  and as multifunctional cytokine exerts its biological activities through two molecules: IL-6R $\alpha$  (IL-6 receptor) and gp130R $\beta$ . When IL-6 binds to mIL-6R $\alpha$  (membrane-bound form of IL-6R), homodimerization of gp130 $\beta$  is induced and a high-affinity functional receptor complex of IL-6, IL-6R $\alpha$ , and gp130 $\beta$  is formed. Interestingly, sIL-6R (soluble form of IL-6R) also binds with IL-6, and the IL-6-sIL-6R complex can then form a complex with gp130 $\beta$

[117]. The complex activates JAKs that then phosphorylate tyrosine residues in the cytoplasmic domain of gp130 $\beta$ . The gp130 $\beta$ -mediated JAK activation by IL-6 triggers two main signaling pathways: the gp130 $\beta$  Tyr759-derived SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase-2)/ERK (extracellular signal-regulated kinase) MAPK (mitogen-activated protein kinase) pathway and the gp130 $\beta$  YXXQ-mediated JAK/STAT pathway [171]. In contrast to LRb, gp130 $\beta$  has more tyrosines for phosphorylation (Y<sup>767, 814, 905, 915</sup>) which is functionally important to negate feedback mechanism mediated by suppressor of cytokine signaling (SOCS) proteins [205]. SOCS3 binds to phosphorylated LRb through its SH2 domain to stop JAK-mediated phosphorylations [22] and additionally recruits ubiquitin-transferases for JAK/STAT degradation [2]. Consequently, leptin-dependent regulation of skeletal muscle is transient, whereas IL-6 seems to induce long-term effects. It was noticed that both cytokines possibly stimulate AMP-activated protein kinase (AMPK) by T<sup>172</sup> phosphorylation mediated by AMPK kinase (LKB1) [213]. Alternatively, by increased cellular ATP turnover, leptin and IL-6 may activate AMPK with elevated levels of allosteric activator AMP. This system is fundamental for energy sensing and fatty acid oxidation in skeletal muscle, so leptin and IL-6 are mighty directors in delivery of substrates for energy production (acetyl-CoA). Recently, we observed other metabolic effects of leptin. In cultured C2C12 mouse myogenic cell line, this cytokine stimulated myoblast mitogenicity and inhibited muscle cell differentiation through JAK/STAT and MEK signaling pathway where MEK is an upstream ERK kinase (MAPKK) [145, 146]. Leptin through MEK-dependent manner caused GSK-3 $\beta$  phosphorylation (Y<sup>216</sup>-GSK-3 $\beta$ ) with resultant drop in myoblast viability and fusion. Overall, more research on adipokines is required in order to shed light on fat-to-muscle influences.

## 5.8 Conclusions and Perspectives

Skeletal muscle fibers as permanent cells do not divide, so upon injury this organ regeneration is entirely reliant on stem cell subpopulations including satellite cells which are activated by a myriad of signals where cytokines play the prime role. Nowadays, it is widely accepted that cytokines which were initially specific growth/differentiation factors of leukocyte origin are also synthesized and secreted by other tissues including skeletal muscle (myokines). It appears, that myokine profile is dependent on the physiologic (type and intensity of exercise, muscle fiber type) or pathologic (the magnitude of injury) conditions. Thus, it is obvious the pattern of myokines controls skeletal muscle growth and decline in health and disease. Skeletal muscle growth is regulated by mitogenic and survival signals (HGF/SF, FGF, IGFs, LIF), whereas damaged skeletal muscle is characterized by necrosis and activation of pro-inflammatory (TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-6) followed by anti-inflammatory cytokines (IL-1ra, soluble TNF- $\alpha$  receptors (sTNF-R), and IL-10) targeting muscle satellite cells and controlling skeletal muscle degeneration

and regeneration phase, respectively. Finally, TGF- $\beta$  superfamily of cytokines, greatly MSTN, stops further growth and muscle tissue regeneration. As skeletal muscle satellite cells are decisive for skeletal muscle regeneration, understanding the molecular mechanisms of their activation by myokines seems to be essential to combat aberrant muscle growth and repair.

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